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GIBBERELLIC ACID INDUCES ASYMBIOTIC GERMINATION OF THE OBLIGATE MYCOHETEROTROPH *PTEROSPORA ANDROMEDEA* (ERICACEAE)

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Abstract

Studies of the Monotropoideae (monotropes; Ericaceae), a monophyletic group of non-photosynthetic, mycoheterotrophic, and often rare or endangered plants, have been limited by the inability to propagate them. Monotropes associate with specific fungal hosts, and the only previously known method of seed germination was induction by host fungi or closely related fungi. In order to overcome very low monotrope seed germination rates and to facilitate further study and conservation efforts, we developed a method using gibberellic acid (GA) to induce asymbiotic germination. *Pterospora andromedea* Nutt. (Monotropoideae, Ericaceae) and *Sarcodes sanguinea* Torr. (Monotropoideae, Ericaceae) seeds from California were exposed to their fungal symbiont or to agarose infused with 0–1 mM GA and then scored for germination. Continuous exposure to 0.5 mM GA for two months induced 75% *P. andromedea* germination, compared to only 21% with its host fungus, *Rhizopogon salebrosus* A.H. Sm. (Basidiomycota). Even short GA exposure (one or three days) significantly enhanced germination (69% and 90%, respectively). The highest germination rate was observed with exposure to 0.5 mM GA for three or 14 d. The closely related *S. sanguinea* required a three-month exposure to GA and even then produced far lower germination rates (~1%). Nevertheless, this is the only known method of inducing monotrope germination without the presence of a specific fungal symbiont. In the case of *P. andromedea*, exogenous GA stimulates germination at rates far higher than that achieved with its fungal symbiont. Application of GA to induce monotrope germination may be used to examine the early stages of mycoheterotroph development, to improve assays for seed viability, and potentially to aid conservation efforts.

Key Words: Gibberellic acid, monotropes, mycoheterotroph, *Pterospora*, *Sarcodes*, seed germination.

Mycoheterotrophic plants are non-photosynthetic parasites of fungi, and they are represented by over 400 species in 87 genera (Leake 1994). Some mycoheterotrophs such as the Monotropoideae are epiparasites that indirectly parasitize surrounding plants through their mycorrhizal fungi in a tripartite symbiosis. In the classical ectomycorrhizal symbiosis, a photosynthetic plant fixes carbon from the atmosphere and trades a portion of its carbon to a fungus growing on its roots in exchange for mineral nutrients. Epiparasites also form associations with mycorrhizal fungi, but these mycoheterotrophs receive carbon from rather than donate carbon to their mycorrhizal fungal partner. Thus, epiparasitic plants reverse the normal flow of carbon found in typical mycorrhizal interactions (Björkman 1960) and represent an extreme in the continuum of plant–fungal interactions within mycorrhizal symbioses (Smith and Read 2008).

The Monotropoideae (Ericaceae, hereafter monotropes) have drawn scientific interest since the birth of mycorrhizal studies (Frank 2004 translation of 1885 paper), and with the advent of molecular techniques, monotropes continue to inspire new lines of investigation (Berch et al. 2005; Merckx et al. 2009; Braukmann and Stefanović 2012; Ogura-Tsujita et al. 2012). Individual monotrope species have been found to be highly specialized on single genera, species groups, or species of ectomycorrhizal fungi (Bidartondo and Bruns 2001, 2002, 2005). The monotropes as well as many other epiparasitic plants are often rare or endangered (Wallace 1975; Wogen and Lippert 1998; Schori 2002; Brown et al. 2003; Lok et al. 2009) and represent uniquely difficult conservation targets due to their dependence on specific host fungi and the autotrophic plants with which their fungi associate.

Our study utilized material from California *Pterospora andromedea* Nutt. populations to investigate seed germination in this monotypic species. *Pterospora andromedea* has a range spanning the continental United States, southern Canada, and Mexico (Wallace 1975). However, this broad range consists of two discontinuous populations. The Eastern population, with a recorded range from southeastern Canada through the northeastern United States, is rare and in many locales endangered (Schori 2002).

The Western population ranges from Mexico north to Canada and east to the Rocky Mountains, and within at least parts of this western range the plant can be locally abundant. Throughout its range, *P. andromedea* associates with host fungi in the genus *Rhizopogon* subgenus *Amylopogon* (Basidiomycota). In the West, *R. salebrosus* A.H. Sm. or *R. arctostaphyli* A.H. Sm. appear to be the primary hosts (Bidartondo and Bruns 2002), and there is a single report with *R. ellenae* A.H. Sm. (Dowie et al. 2011). In eastern North America, Hazard et al. (2011) found that a related, but undescribed, *Rhizopogon* species serves as the host for *P. andromedea*. This eastern species is less common than *Rhizopogon* in western forests and may be limiting the range and frequency of *P. andromedea* in the East (Hazard et al. 2011).

Evolution by angiosperms of diverse mechanisms for maintaining dormancy reflect adaptations to particular environmental conditions (Finch-Savage and Leubner-Metzger 2006). A requirement for the presence of its host fungus is one such adaptation that enables *P. andromedea* to avoid breaking dormancy under unfavorable conditions. *Pterospora andromedea* has inflorescences up to a meter tall and is one of the largest monotropes. However, *P. andromedea* has tiny, dust-like seeds that contain few nutritional resources, and seedlings are unlikely to survive for long without a suitable host fungus (Bakshi 1959; Bruns and Read 2000). Bakshi (1959) failed to germinate seeds under a wide variety of temperature, storage, and nutrient conditions, including planting the seeds in soil collected from the root zone of *P. andromedea* and from soil collected from the root zone of *Picea pungens* Engelm.. He used a tetrazolium method to determine seed viability and reported that no seeds older than nine weeks appeared viable (Bakshi 1959). Using just fungal cultures isolated from the plants, Bruns and Read (2000) discovered that seeds could be induced to germinate at low levels even when substantially older than nine weeks. Then, using a broader sampling of fungi, they demonstrated that only members of the *Amylogogon* subgenus of *Rhizopogon* induced germination. Each mature plant demonstrates even greater host specificity and only associates with a single species within the same subgenus of fungi identified by the germination experiments (Bidartondo and Bruns 2002; Hazard et al. 2011).

A complex balance of hormones controls seed germination and the termination of dormancy in plants (Kucera et al. 2005). The importance of gibberellins (GAs) and their activity as promoters of seed germination is well documented in model systems (Koornneef et al. 2002; Sun and Gubler 2004). GA has been applied to many non-model plants for conservation (Li et al. 2007; Ortega-Baes and Rojas-Aréchiga 2007; Flores et al. 2008; Zeinalabedini et al. 2009; Mattana et al. 2012; Kandari et al. 2012) and restoration (Commander et al. 2009; Turner et al. 2012). However, the efficacy of GA on monotrope germination has not been examined. The inability to grow *P. andromedea* from seed severely limits propagation efforts as well as further experimental work. Here we present the only known method for asymbiotic germination of *P. andromedea* seeds using gibberellic acid (GA), as well as evidence that GA may be used to germinate other monotropes such as *Sarcodes sanguinea* Torr.

Materials and Methods

Fungal Strains and Seed Collections

Pterospora andromedea seeds and roots were collected from Blodgett Forest, a University of California research station (38°54′N, 120°39′W, elev. ~1370 m) situated in the Sierra Nevada foothills near Georgetown, CA. In order to identify the fungal host of individual *P. andromedea* plants, small root fragments were collected in summer (August 2004, July 2005, and August 2008), after emergence of inflorescences. Roots were stored on ice or at 4°C prior to DNA extraction. Genomic DNA was extracted from colonized rootball tissue using the XNAP RED-Extract-N-Amp™ Plant PCR Kit (Sigma-Aldrich, St. Louis, MI), and the internal transcribed spacer (ITS) region from the host fungus was PCR amplified using primers ITS1F and ITS4B (Gardes and Bruns 1993). PCR products were sequenced at the UC Berkeley DNA Sequencing Facility using ABI chemistry. Sequences were identified as either *R. salebrosus* or *R. arctostaphyli* by matching to sequences of known specimens in the GenBank database (Bidartondo and Bruns 2001, 2002). Mature *P. andromedea* seeds from 21 of these fungal host-typed plants were collected after seed set in November 2004, September 2005, and October 2008 and stored at 4°C. Only *P. andromedea* seeds from plants associated with *R. salebrosus* in the field were used in this work.

All seed germination experiments involving the host fungus used *R. salebrosus* strain TDB-379, which was isolated from *P. andromedea* roots, and grown on Modified Melin Norkrans (MMN) medium with 1.5% agar (Bruns and Read 2000).

Mature *Sarcodes sanguinea* seeds were collected in August 2008 from eight plants near the USDA Forest Service work station (37°03′N, 119°9′W, elev. 1375 m) near Dinkey Creek, CA in the Sierra National Forest. All seeds were stored at 4°C prior to use.

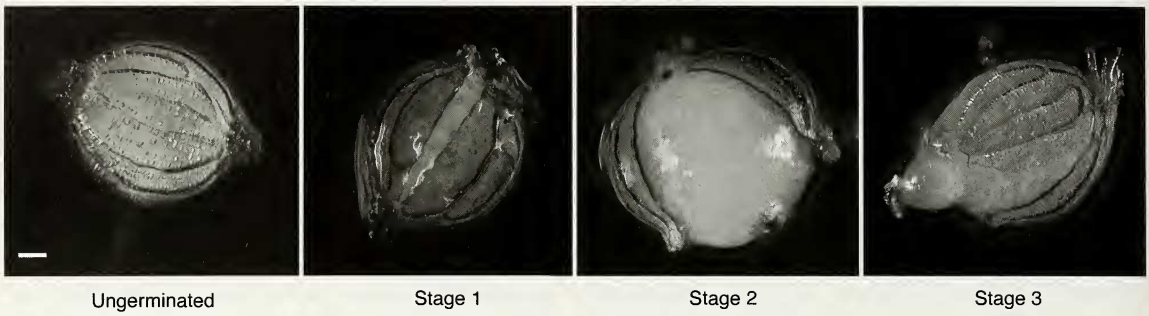

FIG. 1. Stages of *Pterospora andromedea* seed germination. All seeds were de-winged and placed on agarose with gibberellic acid. Ungerminated seeds have intact seed coats. Stage one seeds have cracked seed coats. Stage two seeds have imbibed and swelled. Stage three seeds have visible radicle emergence. Scale bar denotes 100 um.

Gibberellic Acid Assays

Wings were removed from *Pterospora andromedea* seeds less than one year after collection. Seeds were surface sterilized by gentle agitation for 20 min in saturated calcium hypochlorite with a drop of Tween 80, then filtered onto sterile Whatman paper, and rinsed twice with sterile water (Bruns and Read 2000). Seeds were plated onto 2% water agar and monitored for one week for contamination. Contaminated seeds were excised and discarded. Seeds were manually transferred to 0.8% agarose with 0.01, 0.1, 0.5, or 1 mM filter-sterilized GA (Sigma-Aldrich, St. Louis, MI), or to agarose without GA as a disruption control. Seeds exposed to *R. salebrosus* were placed just ahead of the growing mycelial front. After two months, germination was assessed according to the stages described by Bruns and Read (2000) (Fig. 1). Experiments used at least 100 seeds of each germination treatment (exposure to *R. salebrosus*, or combination of GA concentration and time exposed to GA) per replicate.

Statistical Analysis

Percent germination was calculated for replicates of each combination of germination treatment, time, and stage of germination. For experiments with continuous GA exposure, one-way ANOVAs were run for stage three and total germination, with treatment condition as the independent variable. For experiments where seeds were exposed to GA and then transferred to plain agarose, two-way ANOVAs were done for percent total germination and stage three germination. The factors were GA concentration and time of GA exposure, with Tukey HSD tests on the time factor. Statistical analyses were done with JMP software version 5.0.1a for Mac (SAS Institute, Inc, Cary, NC).

RESULTS

Continuous exposure to GA induced germination in *P. andromedea* (Fig. 2). Germination treatment (either GA concentration or exposure to host fungus, *R. salebrosus*) had a significant effect (one-way ANOVA, $P < 0.0001$) on both total and stage three percent germination (Table 1). According to Tukey HSD tests, there was a significant decrease in stage three germination with GA concentrations over 0.1 mM, even though total germination increased. Optimal total germination response of 75 ± 10% (mean ± SD) germination occurred with 0.5 mM GA, which was significantly higher than 0% germination on agarose alone, germination with 0.01 mM GA (0.8 ± 2.4%), or germination with the host fungus, *R. salebrosus* (21 ± 12%). Stage three germination, indicated by radicle emergence, was highest with 23 ± 14% germination on 0.1 mM GA. Stage three germi-

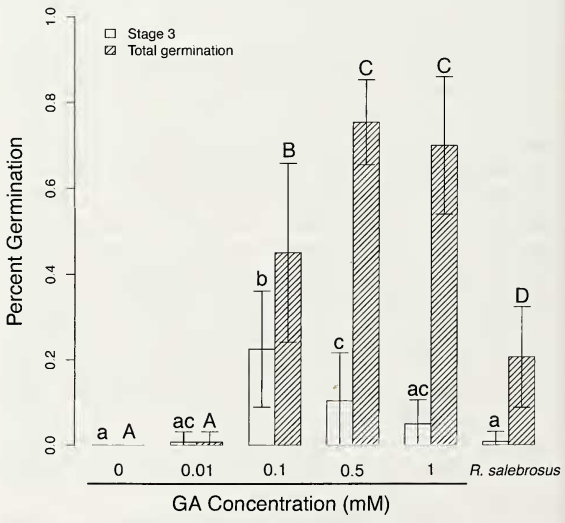

FIG. 2. Gibberellic acid induces *Pterospora andromedea* seed germination better than its host fungus, *Rhizopogon salebrosus*. Error bars indicate standard deviations. Lower case and capital letters respectively indicate significance levels based on Tukey HSD tests for stage three (radicle emergence) and total germination, with treatment condition as the independent variable and a 95% confidence.

TABLE 1. RESULTS OF ONE-WAY ANOVAS DETAILING THE EFFECTS OF CONTINUOUS EXPOSURE FOR TWO MONTHS TO GA ON PERCENT TOTAL GERMINATION AND STAGE THREE GERMINATION OF *PTEROSPORA ANDROMEDEA* SEEDS.

Total germination					
Source	df	Sum of squares	Mean square	F	P
Germination treatment	5	4.561	0.912	59.394	<0.0001
Error	48	0.737	0.015		
Stage 3 germination					
Source	df	Sum of squares	Mean square	F	P
Germination treatment	5	0.321	0.064	12.368	<0.0001
Error	48	0.249	0.005		

nation induced by *R. salebrosus* was significantly lower (0.9 ± 2.4%).

A short exposure to GA (Fig. 3) was sufficient to induce *P. andromedea* germination and outperformed continuous exposure for stage 3 germination (Fig. 2). Time on GA, GA concentration, and the interaction of time and concentration significantly affected total and stage three percent germination (two-way ANOVA, $P < 0.0001$, Table 2). Tukey HSD tests indicated that three days of exposure to 0.5 mM GA was not significantly different from a 14-day exposure while still showing an improvement over a one-day exposure. Three days of exposure to 0.5 mM GA led to 90 ± 0% total germination response and 68 ± 8% stage three germination. Two weeks of GA exposure produced 93 ± 11% stage three germination. Parallel treatments with 0.1 mM GA showed significantly less germination for one- (2 ± 4%) and three- (8 ± 8%) day exposures, while a two-week exposure produced 72 ± 11% stage three and 77 ± 11% total germination. After two weeks of GA exposure, total germination, but not stage three germination, was significantly different between 0.1 and 0.5 mM GA. The total germination responses to each of the three GA exposure times were significantly different from each other. Stage three germination with 14 d of GA exposure was significantly different from just one or three days' exposure.

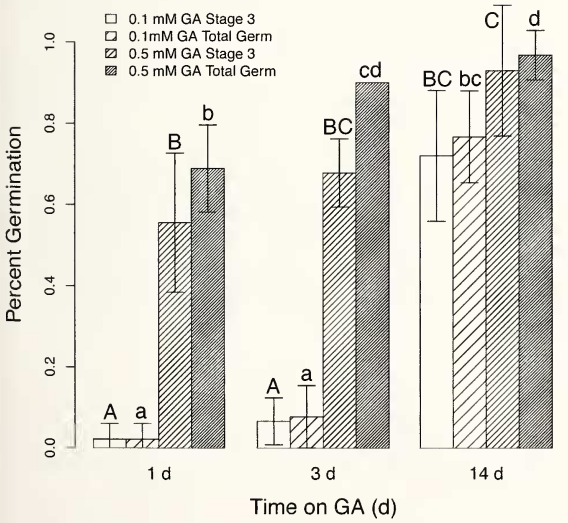

FIG. 3. Short exposure to gibberellic acid induces *Pterospora andromedea* seed germination. Seeds were exposed to gibberellic acid (GA) and then transferred to unamended agarose. Error bars indicate standard deviations. Lower case and capital letters respectively indicate significance levels based on Tukey HSD tests for stage three (radicle emergence) and total germination.

Sarcodes sanguinea seeds failed to respond to GA concentrations of 0.01, 0.1, 0.5, and 1 mM after two months. However, after three months low levels of germination occurred with 0.5 mM and 1 mM GA (1 ± 1% and 1 ± 0.7%, respectively). In the presence of *R. salebrosus*, 42 ± 14% of the seeds germinated. All germination was stage three.

DISCUSSION

We utilized *P. andromedea*'s germination response to GA to produce an improved method to assay seed viability and to enable further studies and conservation efforts using *P. andromedea* seeds. Continuous exposure to GA led to far higher germination response than with *R. salebrosus*, *P. andromedea*'s host fungus (Fig. 2). Gibberellic acid–induced germination was also higher than the most *P. andromedea* germination observed by Bruns and Read (2000) of 26 ± 21% (SD). The ability of GA to germinate seeds well beyond nine weeks old indicates that it is a more accurate determination of seed viability than the tetrazolium method applied by Bakshi (1959). However, while continuous exposure to increasing concentrations of GA did lead to higher overall germination response, increased GA concentrations also had less stage three germination (Fig. 2), suggesting either inhibition by or toxicity of GA.

This suppressive effect of continuous exposure to GA at higher concentrations was ameliorated

TABLE 2. RESULTS OF TWO-WAY ANOVAS DETAILING THE EFFECTS OF SHORT EXPOSURE TO GA (1 D, 3 D, OR 14 D) ON PERCENT TOTAL GERMINATION AND STAGE THREE GERMINATION OF *PTEROSPORA ANDROMEDEA* SEEDS.

Total germination					
Source	df	Sum of squares	Mean square	F	P
Time exposed to GA	2	1.12	0.560	90.789	<0.0001
GA concentration	1	1.703	1.703	276.253	<0.0001
Time x concentration	2	0.455	0.228	36.910	<0.0001
Error	18	0.111	0.006		
Stage 3 germination					
Source	df	Sum of squares	Mean square	F	P
Time exposed to GA	2	1.389	0.695	36.723	<0.0001
GA concentration	1	1.093	1.093	57.800	<0.0001
Time x concentration	2	0.199	0.0995	5.267	0.0159
Error	18	0.341	0.019		

by exposing seeds to GA for shorter periods followed by transfer to plain agarose. A concentration of 0.1 mM GA required two weeks of GA exposure to get more than 10% germination. Just one day with 0.5 mM GA induced over 4.5 times as much total germination as interaction with *R. salebrosus*, and most of this germination was stage three. Three days of exposure to 0.5 mM GA produced nearly 90% total germination, and two weeks led to nearly all seeds reaching stage three germination. To our knowledge, GA is not known to inhibit germination at higher concentrations. For instance, Fennimore and Foley (1998) found ~90% germination of *Avena fatua* L. after exposure to 10 mM GA.

Sarcodes sanguinea exhibited low germination response to up to 1 mM GA. The seeds were viable, as demonstrated by their germination in the presence of *R. salebrosus*, at a rate very close to the 46 ± 22% found by Bruns and Read (2001). *Pterospora andromedea* seeds are far smaller and have more delicate seed coats than those of *S. sanguinea*. The low germination in *S. sanguinea* could be due to a different porosity or composition of the seed coats or lower sensitivity to GA.

Bruns and Read (2000) found that in vitro, *P. andromedea* germination did not require direct contact with the host fungus, suggesting that a diffusible substance induces germination. The fact that gibberellins were first isolated from a fungus (*Fusarium fujikuroi* Nirenberg) and have since been identified as a secondary metabolite in many other fungi (Bömke and Tudzynski 2009) might lead one to think that GA is the diffusible substance observed by Bruns and Read (2000). However, this is unlikely for two reasons. First, in order for GA to be the diffusible substance, one would expect it to be limited to *R. salebrosus* and *R. arctostaphyli* in order to explain the observed specificity. Yet, as mentioned above, GA is known to be produced by a wide range of unrelated fungi. Second, if GA were the diffusible signal from *Rhizopogon*, one would expect *P. andromedea* and *S. sanguinea* to respond to it in similar ways, yet their sensitivity to it is quite different and uncorrelated with their response to the unknown diffusible substance(s) from *Rhizopogon*. For these reasons, it seems more likely that the chemical signal of *Rhizopogon* either triggers the endogenous gibberellin pathway in *P. andromedea* or works by some independent pathway.

Gibberellic acid–induced germination of *P. andromedea* seeds will clearly provide a useful tool for assessing the viability of seed lots, but whether it can be applied to help establish seedlings is yet to be determined. The main problem is that the next developmental steps are likely to require a compatible association with *Rhizopogon*, its host fungus, which in nature is mutualistically associated with pine roots. Under Petri dish conditions, growth of *Rhizopogon* is much more limited, and germinating *P. andromedea* seeds rarely develop beyond stage three, even when induced to germinate by its host fungus (Bruns and Read 2000). Thus, in order to test whether further development is possible it will probably require a three-organism system to be assembled, and this feat has yet to be achieved with any member of the Monotropoideae. Further work to incorporate GA into a practical conservation plan for *P. andromedea* and other monotropes may require field experiments.

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